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(71)(72) Applicants and Inventors: GUSS, Bengt [SE/SE]; Dag Hamarskjölds väg 238B, S-756 52 Uppsala (SE). JONSSON, Hans [SE/SE]; Börjegatan 58C, S-752 29 Uppsala (SE). LINDBERG, Martin [SE/SE]; Komvägen 5, S-752 57 Uppsala (SE). MÜLLER, Hans-Peter [DE/SE]; Tjälinge 9, S-740 20 Brunna (SE). RANTAMÄKI, Liisa, K. [FI/FI]; Ojahaanpolku 6 B 20, FIN-016 00 Vantaa (FI).

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(54) Title: METHOD AND MEANS FOR PRODUCING PLASMAPROTEINASE INHIBITOR-BINDING PROTEINS

(57) Abstract

Method based on recombinant DNA molecules for producing proteins, or fragments thereof, having binding activity for the fast form of α_2-macroglobulins as exemplified for the streptococcal surface proteins MIG, MAG and ZAG.

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Method and Means for producing Plasmaproteinase Inhibitor-Binding Proteins

The invention relates to the field of gene technology and is concerned with recombinant DNA molecules, which contain nucleotide sequences coding for different proteins or polypeptides which have the ability to specifically bind to α_2-macroglobulin (α_2M), a plasma proteinase inhibitor. The invention also comprises microorganisms containg the aforesaid molecules, and the use thereof in the production of the aforesaid proteins or polypeptide.

The existence of bacteria that bind specifically to the plasmaproteinase inhibitor α_2M has earlier been reported (Müller and Blobel 1983;1985). The binding of α_2M to streptococci is highly specific and streptococci of different serological groups or species bind the two different conformational forms of this plasma proteinase inhibitor referred to as "slow" (sα_2M) and "fast" (fα_2M) based on their electrophoretical behaviour (Müller and Blobel 1983; 1985). The slow form represents the native inhibitor and the fast form the inhibitor/protease complex (Barret et al. 1979). In 1989 Sjöbring et al. reported that both conformational forms of α_2M could be bound to the protein G. This receptor is one of the best studied so called type III Fc receptors (Björck and Åkerström 1990, Sjöbring et al. 1989a, Sjöbring el al. 1991). The gene encoding protein G has been cloned and sequenced (Guss et al. 1986; Olsson et al. 1987) and binding properties been studied (Guss et al. 1986; Björck et al. 1987; Åkerström et al. 1987; Nygren et al. 1988). As a result of these studies it has been shown that protein G binds both IgG and serum albumins through specific domains in the protein. Sjöbring et al. 1989b reported that in addition of binding these two serum proteins protein G should also bind to both conformational forms of α_2M. The latter binding they reported should be located in the IgG binding domain of protein G, - a finding that will be contradicted in the present application. The quantification of free α_2M and α_2M-protease complexes using streptococcal α_2M receptors have been described (Justus

et al. 1990). Also in studies concerning purification and characterisation of α_2M from mastitis milk these streptococcal receptors have been used (Rantamäki and Müller 1992). In these cases intact streptococcal cells have been used as the source of the receptor. Therefore should a protein which for instance binds specifically to α_2M be of great biotechnological interest. This protein could in analogy to what Nygren et al. 1988 reported be immobilized and used to affinity purify away any undesired α_2M in the sample. Furthermore different proteins (or fragments thereof) with α_2M-binding activity could be used to detect and measure the amount of α_2M in a sample. Generally it may be difficult to obtain a homogeneous and reproducible product if such receptors should be prepared from streptococcal cells directly. Moreover, most streptococci are pathogenic and need complex culture media which involve complications in large-scale cultures. There is thus a need for a new method for producing α_2M-binding proteins (or fragments thereof).

The present invention relates to different recombinant DNA molecules comprising nucleotide sequences which code for proteins or polypeptides having α_2M-binding activity. The natural source of the nucleotide sequences encoding the α_2M-binding activity is the *S. dysgalactiae* strain SC1 and/ or 8215 respectively, and or the *Streptococcus equi* subsp. *zooepidemicus* strain V, but with the knowledge of the respective nucleotide and the deduced amino acid sequences presented here the gene(s) or parts of the gene(s) can be made synthetically or reisolated. In particular the knowledge of the deduced amino acid sequence covering the part of the gene encoding the α_2M-binding activity can be used to produce (synthetical) polypeptides which retain or inhibit the α_2M-binding. These polypeptides can be labelled with various compounds such as enzymes, flourescent dyes, biotin (or derivatives thereof), radioactivity etc. and used in diagnostic tests such as Elisa or Ria techniques.

For producing a recombinant DNA molecule according to the invention a suitable cloning vehicle or vector, for example a plasmid or phage DNA, may be cleaved with the aid of restriction enzymes whereupon the DNA sequence coding for the

desired protein or polypeptide is inserted into the cleavage site to form the recombinant DNA molecule. This general procedure is known per se, and various techniques for cleaving and ligating DNA sequences have been described in the literature (see for instance US 4,237,224; Ausubel et al. 1991; Sambrook et al. 1989); but to our knowledge these techniques have not bee used in the present protein system. If the *S. dysgalactiae* strains SC1, 8215, or the *S. equi* subsp. *zooepidemicus* are employed as the source of the desired nucleotide sequences it is possible to isolate said sequences and to introduce them into suitable vectors in a manner such as described in the experimental part below or since the nucleotide sequences are presented here use a polymerase chain reaction (PCR)-technique to obtain the complete genes or fragments of the *mig*, *mag*, or *zag* genes.

Hosts that may be employed- that is, microorganisms which are caused to produce the proteins or active fragments thereof - may comprise bacterial hosts such as strains of e. g. *Escherichia coli*, *Bacillus subtilis*, *Streptococcus* sp., *Staphylococcus* sp., *Lactobacillus* sp. and furthermore yeasts and other eucaryotic cells in culture. For obtaining maximum expression, regulatory elements such as promoters and ribosome-binding sequences may be varied in a manner known per se. The protein or active protein thereof can be produced intra- or extracellular. To obtain good secretion in various bacterial systems different signal peptides can be employed. To facilitate the purification and/or detection of the protein or fragment thereof, they could be fused to an affinity handle and /or enzyme. This can be done on both genetical and protein level. To modify the features of the protein or polypeptide thereof the gene or parts of the gene can be modified by e.g. in vitro mutagenesis; or by genefusion with other nucleotide sequences which encode polypeptides resulting in fusion proteins with new features.

The invention thus comprises recombinant DNA molecules containg a nucleotide sequence which codes for a protein or polypeptide having a α_2M-binding activity. Furthermore, the invention comprises vectors such as plasmids and phages containing such a nucleotide sequence, and organisms,

especially bacteria e.g. strains of *E.coli*, *B.subtilis* *Streptococcus* sp. and *Staphylococcus* sp., into which such a vector has bee introduced. Alternatively, such a nucleotide sequence may be integrated into the natural gene material of the microorganism.

The application furthermore relates to methods for the production of proteins or polypeptides having the α_2M-binding activity of protein MIG, MAG, ZAG or active fragments thereof. According to this method, a microorganism as set forth above is cultured in a suitable medium whereupon the resultant product is isolated by affinity chromatographic purification with the aid of IgG or albumin bound to an insoluble carrier, or by means of some other separating method, for example ion exchange chromatography.

Vectors, especially plasmids, which contain the respective protein MIG, MAG or ZAG encoding nucleotide sequences or parts thereof may advantageously be provided with a readily cleavable restriction site by means of which a nucleotide sequence that codes for another product can be fused to the respective protein MIG, MAG or ZAG encoding nucleotide sequence, in order to express a so called fusion protein. The fusion protein may be isolated by a procedure utilising its capacity of binding to fα_2M and/ or IgG, whereupon the other component of the system may if desired be liberated from the fusion protein. This technique has been described at length in WO 84/03103 with respect to the protein A system and is applicable also in the present context in a analogous manner. The fusion strategy may also be used to modify, increase or change the fα_2M binding activity of the MIG, MAG and ZAG proteins or fα_2M binding parts thereof by combining them in various combinations or with other α_2M binding proteins.

Starting materials

Bacterial strains and cloning vectors

Streptococcus dysgalactiae strain 8215 and *Streptococcus equi* subsp. *zooepidemicus* strain V were obtained from The National Veterinary Institute, Uppsala, Sweden. The *S. dysgalactiae* strain SC1 was obtained from Dr Rantamäki, Dep. of

Microbiology and Epizoology, College of Veterinary Medicine, Helsinki, Finland.
E.coli strain DH5α was used as bacterial host for the plasmids to be constructed. *E. coli* strain P2392 was used in cloning with the lambda vector EMBL3 (Frischauf et al., 1983) and as a host for expression of the lambda SD1 or lambda SZG1 encoded plasmaprotein-binding proteins, termed protein MAG and protein ZAG.
The lambda EMBL3 vector, *E. coli* strain P2392 and in vitro packaging extract (Gigapack ®II gold) were otained from Stratagene, La Jolla, CA, USA. The plasmid vector pGEM11zf(+) was obtained from Promega, Madison,WI. USA. A protein fusion and purification system obtained from NewEngland Biolabs, USA was used to construct clones expressing various parts of the *mig, mag* or *zag* genes fused to the vector pMALC2. This system was used to construct the expression clones pMIG1-3 pMAG1-4, and pZAG1-2. The system was used essentially according to the manufacturers recommendations but the host strain was *E. coli* strain DH5α.

All strains and plasmid- or phage-constructs used in the examples are available at the Department of Microbiology at the Swedish University of Agricultural Sciences, Uppsala, Sweden.

Buffers and media

E. coli was grown on LB (Luria Bertani broth) agar plates or in LB broth (Sambrook et al., 1989) at 37°C. Ampicillin was in appropriate cases added to the E. coli growth media to a final conc. of 50 µg/ml. The streptococci were grown at 37°C on bloodagar-plates (containing 5% final conc. bovine blood) or in Todd-Hewitt broth (obtained from Oxoid, Ltd Basingstoke, Hants., England) supplemented with Yeast Extract (Oxoid) to a final conc. of 0.6%. PBS: 0,05M sodium phosphate pH 7,1; 0,9 % NaCl. PBS-T: PBS supplemented with TWEEN 20 to a final conc. of 0,05%.

Preparation of DNA from Streptococci

Streptococci of resp. strains and species were grown separately overnight in Todd-Hewitt Broth supplemented with 0.6% yeast extract and 10 mM glycine. The next morning glycine was added to a conc. of 0.067 M and the incubation was continued at 37°C for additional 2h. After harvest the cells were washed three times in a buffer consisting of 50 mM Tris-HCl pH 7.0 + 50 mM EDTA and resuspended to a 1/20 of the original culture volume in the same buffer including 25% sucrose. Lysozyme (Boehringer, Germany) was added to a final conc. of 30 mg/ml and the suspension was incubated with gentle agitation for 2h at 37°C. The cells now converted to protoplasts were then pelleted by centrifugation and resuspended in buffer consisting of 50 mM Tris-HCl pH 7.0 + 50 mM EDTA including 1% SDS (sodium dodecyl sulphate) and incubated at 65°C for 15 min. Cell debris was removed by centrifugation and the viscous supernatant further treated as described for chromosomal DNA preparations (Sambrook et al. 1989).

Proteins and other reagents

Human IgG (Gammaglobin, KabiVitrum, Stockholm) was absorbed to protein A Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden), and after elution with glycine HCL buffer, pH 2.8, immediately neutralised and then dialysed against PBS, pH 7.4. Human α_2M and bovine α_2M were purified on the day of bleeding from fresh acid citrate dextrose plasma by polyethylenglycol precipitation, gel filtration and affinity chromatography as recently described for bovine α_2M (Rantamäki and Müller, 1992). Human IgG and human α_2M converted by trypsin to the fast form, were iodinated by the chloramine T method (Hunter, 1978) to a specific activity of 125 MBq/mg. The preparation of the bovine α_2M peroxidase conjugate (α_2M in the fast form) has recently been described (Rantamäki and Müller, 1992). Goat albumin (Sigma) was conjugated with peroxidase (Boehringer, Mannheim, FRG) in a molar ratio of 1:2 according to Wilson and Nakane (1978). The goat anti rabbit IgG peroxidase conjugate

(Bio-Rad, Richmond, Ca, USA) was used for detection of the IgG-binding activity. Bovine serum albumin (fraction V, ria grade) was obtained from USB (cat. no.10868).DNA probes were labelled with alpha^{32}P-ATP by a random-priming method (Multiprime DNA labelling system; Amersham Inc, Amersham, England). Nitrocellulose (nc)-filters (Schleicher & Schüll, Dassel, Germany) were used to bind DNA in hybridization experiments or proteins in dot-blot or Western-blot techniques.
In order to analyze protein samples by native or SDS-PAGE the PHAST-system obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden was used according to the suppliers recommmendations.

Routine methods

Methods used routinely in molecular biology are not described such as restriction of DNA with endonucleases, ligation of DNA fragments, plasmid purification etc since these methods can be found in commonly used manuals (Sambrook et al., 1989, Ausubel et al., 1991).

Example 1 (A). Construction and screening of a genomic library from *S. dysgalactiae*, strain SC1

Chromosomal DNA from *S. dysgalactiae* SC1 was partially digested with Sau3A1, and fragments ranging from 3-8 kb in size were isolated by preparative agarose gel electrophoresis. The DNA fragments were ligated to Lambda GEM-11 BamH1 Arms (Promega, Madison, Wi, USA). After ligation the recombinant molecules were packaged in vitro using the Packagene System (Promega). The resulting phage library was screened for expression of IgG- or α_2M-binding activity using ^{125}I-labelled IgG or α_2M, respectively. After generation of plaques on the host strain *E. coli* LE 392, a nc filter (Schleicher & Schüll, Dassel, FRG) was placed on top of the agar plate. The filter was then removed and saturated with 2% BSA in PBS for 1h at 37°C, rinsed briefly in PBS containing 0.05% Tween 20 (PBS-T).

A second filter treated in the same way was used to obtain a second replica of the plate. The two filters were then incubated with ^{125}I-labelled α_2M and IgG, respectively, overnight at room temperature in the same buffer. Subsequently the filters were washed 3 x 15 min in PBS-T, dried and exposed to Kodak XAR5 film for 7 days at -70°C. Plaques that gave a signal with radiolabelled ligands were replaqued and the binding activities verified in a second binding experiment.

Example 1(B) Subcloning and isolation of positive clones

One lambda clone reactive with both ligands, designated A5 was chosen for further studies. The clone was analysed by restriction mapping and a preliminary restriction map was constructed. After *Hin*dIII digestion of the phage A5 DNA, fragments were cloned into pUC18. Following transformation, clones were grown overnight on nc filters on agar plates. The nc-filters were replicated to a masterplate and incubated for 10 minutes in chloroform vapour in order to release the proteins from the bacterial cells. After saturation with BSA the filters were incubated with radiolabelled ligands as mentioned above. Positive clones were isolated after 2 days exposure of the filters to Kodak XAR-5 film. One clone, pAM1, reacted with both α_2M and IgG and was selected for further studies. Subcloning of the DNA into a plasmid vector showed that a 2,5 kb *Hin*dIII restriction fragment encoded both the α_2M and IgG binding.

Example 1 (C). DNA sequencing and analysis of the sequence

The clone pAM 1 was subcloned into pUC18 and pGEM11zf(+)(Promega). The subclones were sequenced by the dideoxy method of Sanger et al.(1977) using "Sequenase, version 2.0" (USB,Cleveland, Ohio, USA). The sequencing samples were analysed on wedge-shaped 6% acrylamide gels containing 7 M urea. The "PC GENE" computer software package

(Intelligenetics Inc., CA, USA) was used for analysis of the DNA sequences.

Sequencing of the *Hind*III fragment revealed an open reading frame starting with an ATG initiation codon in position 506 and ending with a TAA stop translation codon in position 2498, thus coding for a potential protein of 664 amino acids (Page 31). Preceding the ATG codon, in position 494 to 499, there is a putative ribosome binding site (Page 31, double underlined) similar to those found in other streptococcal genes. Further upstream there are stretches, that constitute possible transcription initiation signals (single underlined). The deduced amino acid sequence of the *mig* gene contains an N-terminal stretch resembling a secretory signal. There is a possible cleavage point according to the -1, -3 rule (von Heijne, 1986) in amino acid position 30-31. Following the putative signal sequence there is a 178 amino acid stretch containing several repetitive motifs of various length. For example in position 87-110 there is a 24 amino acid stretch, which is repeated once, starting at position 166. The function of the various repeats has so far not been determined. Further downstream the gene is extremely repetitive. In amino acid position 209, starting with alanine, a stretch of 70 amino acids is repeated five times without any intervening segment and with only minor amino acid substitutions within the elements. Downstream these repeats there is a stretch containing many charged amino acids and many prolines, a feature seen in several other streptococcal surface proteins(Fahnestock et al., 1990). This stretch, probably spanning the cell wall, is called W. Further downstream there is a LPTTGE sequence matching the consensus hexapeptide LPXTGX found in all cell wall associated proteins in Gram-positive cocci. In the C-terminal end of the protein there is a hydrophobic stretch ending with a few charged amino acids, constituting the membrane spanning region called M. Further analysis of the nucleotide and amino acid sequence reveals a striking homology between the C-terminal parts of protein MIG and protein G from group G streptococci (Fahnestock et al., 1986; Guss et al., 1986). The five major repeats from protein

MIG, responsible for the IgG-binding, comprise amino acid 209 to amino acid 559. The region upstream the initiation codon and also the region encoding the main part of the signal peptide are highly homologous in the two genes (data not shown). The region in the SC1 gene from position 587 to the start of the 70 amino acid repeats (in position 1130) shows no apparent homology to any sequence in the EMBL nucleic acid sequence data bank.

Example 1 (D). Construction of expression clones and purification of fusion protein

A protein fusion and purification system (NewEngland Biolabs) was used to construct clones expressing various parts of the *mig* gene fused to the vector pMalC2. The 1860 bp PvuII-PvuII fragment ranging from position 570 to position 2430 represented the whole gene except the signal sequence and the membrane spanning domain (Page 31 and Fig.1). This clone was designated pMIG1. The clone containing the 521 bp PvuII-Sau3A fragment from position 570 to 1091 was called pMIG2. Finally the 1104 bp Sau3A-BstNI fragment ranging from position 1091 to 2195 was cloned and the clone was named pMIG3. The different clones were cultivated in 5 liters of LB medium with 50 µg ampicillin/ml, according to the instructions of New England BioLabs. Cells were harvested by centrifugation and subsequently lysed on ice for 30 min in 50 ml lysis buffer, consisting of 10 mM Na_2HPO_4, 30 mM NaCl, 0.25% Tween 20, 10 mM EDTA, 10 mM EGTA, 1mM PMSF and 1 mg/ml hen egg white lysozyme (Boehringer Mannheim, FRG). The lysates were then extensively sonicated on ice for 2 min to reduce the viscosity and centrifuged for 30 min at 20 000 x g. The clear supernatants were diluted with equal volumes of PBS, containing 0.05% Tween 20 and 0.05% NaN_3. The samples were then loaded on an amylose-agarose column (NewEngland BioLabs) at a flow rate of 2 ml/min and after extensive washings with the start buffer the column was eluted with the same buffer, containing 10 mM maltose. Elution was recorded at 280 nm and fractions of 3 ml were collected.

Example 1 (E). SDS-PAGE and Western blotting

Material from the expression clones was prepared for gel electrophoresis by taking 1 ml of IPTG induced culture, pelleting the cells and resuspending them in sample buffer (Laemmli, 1970) containing SDS and 2-mercaptoethanol. After boiling for 5 min the suspensions were centrifuged in a microfuge and the supernatants applied to the electrophoresis gel in the Mini protean Chamber (Bio-Rad, Richmond, CA, USA). The samples were separated on a 12,5% acrylamide gel and subsequently stained with Coomassie blue R250 (Serva, Heidelberg, FRG) or blotted onto nc-membranes. The nc-membranes were blocked with 1% BSA in PBS and reacted with peroxidase labelled bovine α_2M (Rantamäki and Müller, 1992) and peroxidase labelled goat IgG (Bio-Rad), respectively. After washing with PBST and PBS, the nc membranes were developed with 4-chloro-1-naphthol (Serva) for visualization.

Example 1 (F). Localization of the different binding activities

In order to facilitate the purification of the gene products and to determine the location of the two binding activities, a number of expression clones were constructed using the pMAL gene fusion vector (Fig.1). The reactivity of the different expression clones was tested in Western Blot experiments (Fig.2). The pMIG1 clone, representing virtually the whole *mig* gene, bound both α_2M and IgG, while the pMIG2 clone, which encoded the unique SC1 sequence upstream to the 70 amino acid repeat region, bound only bovine α_2M but neither goat or human IgG nor goat or human albumin. The clone pMIG3, encoding the 5 times repeated 70 amino acid long units, bound only IgG. The two expression clones pMIG2 and pMIG3 were also tested for their ability to inhibit the binding of bovine α_2M and goat IgG to their native receptors present on the surface of SC1 cells (Fig.3). Affinity purified material from the two clones completely inhibited the binding of their corresponding ligand, α_2M or IgG, to the streptococcal cells.

Example 1 (G). Inhibition assay

Flat bottom microtitre plates (Maxisorp, NUNC, Copenhagen, Denmark) were coated with 200 µl of a suspension of guanidinium chloride extracted cells of *S. dysgalactiae*, SC1, as recently described (Rantamäki and Müller, 1992). To demonstrate inhibition of bovine α_2M-binding to the immobilised SC1 cells by the recombinant α_2M-R, 2-fold serial dilutions of the purified fusion protein encoded by pMIG2 were applied to the wells in aliquots of 100 µl and to each well 100 µl of peroxidase labelled bovine α_2M in a predilution of 1:4000 was added. For demonstration of the inhibitory activity of the recombinant FC-R, the fusion protein encoded by pMIG3 was 2 fold serially diluted and 100 µl of each dilution step applied per well. One hundred µl of the peroxidase labelled goat anti rabbit-IgG (BioRad) in a dilution of 1:4000 were then mixed with the fusion protein and the plate allowed to stand for 4h at room temperature. The plate was subsequently washed 5 times with PBS-T and developed using 3,3',5,5',-tetramethyl benzidine (Boehringer) as substrate (Bos et al., 981). Finally the developed blue colour was converted to yellow by addition of 100 µl 1M H_2SO_4 and the plate was read at 450 nm in an ELISA reader (BIOTEK). The result is shown in Fig. 3.

Example 2 (A). Isolation of a lambda clone originating from *S. dysgalactiae* strain 8215 which codes for polypeptides possessing α_2M-, BSA-, and IgG-binding activity

A gene library for *S. dysgalactiae* strain 8215 was produced in a manner analogous to that described by Frischauf et al. (1983).

Streptococcal DNA was partially digested with Sau3A1 and ligated into BamHI-cleaved lambda EMBL3 vector arms. The ligated DNA was packaged in vitro into phage particles which were then allowed to infect *E. coli* P2392 cells. The resultant

phage library was analysed for α_2M-, BSA- and IgG-binding activity. The resultant phage library was analysed for this purpose on agar plates in soft agar layer which were incubated overnight at 37° C. The next day plates having a plaque frequence of 10^3-10^4 were selected. The plaques from each plate were transferred by replica plating to nc-filters. Transfer was allowed to proceed at room temperature for about 15 min. The filters were subsequently removed and soaked, using gentle agitation, for 30 min in a PBS-T solution (250ml/10 filter with three changes of the PBS-T solution in order toremove loosely bound material such as cell debris and components from the growth media). The filters were then sorted into three groups where replicas originating from the same agarplate were represented in each grou. The respective group of filters were transferred to a petri dish which either contained approximately 10^7 cpm of ^{125}I-labelled rabbit-IgG-antibodies (specific actiivity 7 mCi/mg) in PBS-T, approximately 10^7 cpm of ^{125}I-labelled BSA (specific activity 7 mCi/mg) or approximately 10^7 cpm of ^{125}I-labelled fα_2M (specific activity 125 MBq/ml). After incubation for 2h at room temperature using gentle agitation the respective group of filters were washed separately for 3 x 10 min in 250 ml PBS-T at room temperature (this washing procedure is important to reduce background signals and have to be prolonged if necessary). After washing the filters were dried and autoradiographed for several days. Analysis of the autoradiogram revealed several plaques reacting with the three labelled (α_2M, IgG and BSA) ligands. By comparing autoradiograms corresponding to the different ligands clones binding all three ligands were selected on the original agar plate and replaqued and the binding activities verified in an another round of binding assays as described above. Finally one clone called "lambda SD1" expressing all three activities was chosen for the subsequent procedures. Note! The use in biotechnology of the serum albumin binding properties of protein MAG are described in a copending patent application entitled "Method and Means for Producing Plasmaprotein-binding Proteins" filed Sept.6.1993(Nr. 9302856-1).

Example 2 (B). Characterization of the lambda SD1 clone

Purified phage DNA from the lambda SD1 clone was analysed by restriction mapping and a preliminary restriction map was constructed. After HindIII digestion of the lambda SD1 DNA, fragments were cloned into the pGEM11Zf(+) previously cleaved with HindIII. After ligation and transformation into *E. coli* strain DH5α recombinant clones were screened for expression of α_2M-, BSA- and/or IgG-binding activity. This was done as follows: Clones were grown over night on nc-filters on agar plates. Next day the nc-filters were replica plated to a masterplate whereupon the filters were incubated for 10 min in chloroform vapour in order to release the proteins from the bacterial cells. After washing the filters in large excess of PBS-T (in this step it is important to reduce the bacterial debris attached to the filter to avoid unspecific binding of the labelled ligands used in the next step thereby reducing the background signals) the filters were transferred to petri dishes containing ^{125}I-labelled α_2M, BSA, or IgG, respectively as in Example 2A above. After incubation for 2h at room temperature under gentle agitation the filters were washed in PBS-T, dried and autoradiographed as mentioned in Example 2A. After 2 days of exposure of the filters the autoradiograms were analysed and clones expressing α_2M-binding activity were isolated. One such clone called pSD100 harbouring an aproximately 0,9 kb HindIII insert was chosen for further studies. Clones expressing BSA-or IgG-binding activity could not be identified. In order to identify clones encoding the BSA and IgG-binding activity, a ^{32}P labelled DNA (random-priming) probe homologous to the domain encoding the IgG-binding regions of protein G (Guss et al., 1986) was used to identify the presence of homologous sequences among the recombinant plasmid clones. Among several HindIII clones hybridizing to this probe one called pSD101 containing an approximately 0,7 kb insert was selected for further studies.

Example 2 (C). Sequence analysis of the *mag* gene

The nucleotide (nt) sequences of the *Hind*III inserts of pSD100 and pSD101 were determined and the nt and deduced amino acid (aa) sequences were compared with the corresponding sequences from earlier published sequences of type III Fc receptors (Fahnestock et al., 1986, Guss et al., 1986, Olsson et al., 1987). This analysis revealed only short stretches of homology within the 862 bp *Hind*III insert of pSD100 to the other type III Fc receptor genes while the 693 bp *Hind*III insert of pSD101 was highly homologous to the earlier studied receptor genes. The combined size of the directly linked inserts of pSD100 and pSD101 containing the whole gene, called *mag*, is 1555 nt (Fig.4). There is a potential ATG start codon at nt position 288 preceded by a nt sequence resembling a ribosome-binding site. Upstream this site there are several potential promoter sequences. Starting at the ATG codon there is an open reading frame of 1239 nt terminating in a TAA stop codon at nt 1527. Thus the gene encodes a 413 aa protein, termed protein MAG, with a calculated molecular mass of approximately 44 kDa including a putative signal peptide. The N-terminal part of the protein, constituting the signal peptide, shows a high degree of homology to the corresponding domains of the other type III receptors. A possible signal peptidase cleavage site should be after the alanine residue at aa position 34 in Figure 4. Downstream the signal peptide there is a unique strech of 158 aa. No repeated motifs can be seen in this part of the protein from strain 8215. Further to the C-terminal end the deduced aa sequence starting with -ALK- in position 193-195 shows homology to parts of the albumin-binding domain of protein G, the type III Fc receptor from the group G streptococcal strain G148 (Björck et al., 1987, Nygren et al., 1988). In the 8215 receptor this part is only 50 aa long and is directly followed by a region, which is highly homologous to the IgG-binding domains found in other type III Fc receptors. Also in the C-terminal part of the protein, which is responsible for anchorage of the protein to the bacterial cell surface, there is a striking sequence homology to other cell wall associated streptococcal proteins. This part of the

protein can be divided into two structural and functional different domains. The N-terminal part, called W, (Fig.4) is extremely hydrophilic and consists of mainly charged residues and prolines. This part of the protein probably mediates the attachment to the cell wall. Following the W-region, there is a hexapeptide LPTTGE, matching the consensus sequence LPXTGX commonly found in wall achored surface proteins of Gram-positive cocci (Fig.4). Downstream this motif there is a region of hydrophobic residues, called M, spanning the cell membrane, followed by a stretch of positively charged residues in the C-terminal end of the protein.

Example 2 (D). Localization of binding domains in protein MAG.

On the basis of the sequence analysis we used a fusion protein expression system (obtained from Biolabs) to produce the various domains of protein MAG (Fig. 5A). The high homology between the 3' end of the gene and the genes of earlier described type III Fc receptors strongly suggested that this part of the sequence is encoding the IgG-binding activity. In Western-blot experiments we could show that this was indeed the case (see legends to Fig.5A, B and C). The fusion protein encoded by the construct pMAG3 showed no reactivity with α_2M but a strong signal was obtained with labelled albumin and IgG (Figs.5B and C). This construct encodes an IgG-binding domain and also the 50 aa sequence upstream that domain which is partially homologous to the albumin-binding domains of protein G (Björck et al., 1987, Nygren et al., 1988). The subclone pMAG4, encoding the same 50 aa and 10 aa from the IgG-binding domain, was reactive with albumin but not with IgG or fα_2M (Figs. 5B and C). In addition, the subclone pMAG2, encoding the unique 158 aa long stretch in the N-terminal part of the protein reacted only with α_2M. Expression of the clone pMAG1, (representing the whole gene), bound all three ligands.

Example 2 (E). Schematic presentation of the construction of the expression clones pMAG1-4.

pMAG1: lambda SD1 was cleaved with PvuII. The approximately 1.1 kb PvuII fragment representing almost the complete *mag* gene was purified using preparative agarose gel-electrophoresis and ligated into the vector pMALC2 (the vector had previously been cleaved with EcoRI and the sticky ends converted to blunt ends using T4 DNA polymerase.

pMAG2: the 870 bp HindIII fragment from pSD100 was purified by preparative agarose gel-electrophoresis. The purified HindIII-fragment was cleaved by PvuII and a part of the cleaved material was subsequently ligated into the pMALC2 vector (the vector had earlier been cleaved with EcoRI and the generated sticky ends converted to blunt ends using T4 DNA polymerase. After inactivation of the T4 DNA polymerase the vector was cleaved with HindIII).

pMAG3: the 670 bp HindIII fragment from pSD101 was purified by preparative agarose gel-electrophoresis. The purified fragment was cleaved with PvuII and made blunt end with T4 DNA polymerase. The cleaved material was ligated into pMALC2 (the vector had previously been cleaved with EcoRI and BamHI and the sticky ends converted to blunt ends using T4 DNA polymerase.

pMAG4: the 670 bp HindIII fragment from pSD101 was purified using by preparative gel-electrophoresis. The purified fragment was cleaved with HgaI and the sticky ends converted to blunt ends using T4 DNA polymerase and ligated into the pMALC2 vector (the vector had earlier been cleaved with EcoRI and BamHI and converted to blunt ends using T4 DNA polymerase. After ligation and transformation into *E.coli* DH5α the generated clones were screened for binding activities as described above. Clones expressing various binding activities were identified and called pMAG1-4. The presence of the expected inserts in pMAG1-4 were verified by nt sequencing (including nt sequencing over the junctions between vector and insert).

Example 2 (F). Purification of protein MAG using the lambda SD1 clone

The lambda SD1 phages were allowed to adsorb to *E. coli* P2392 cells in 3 individual tubes containing each 3 ml of an overnight culture (37°C, LB-medium with 0.2% maltose and 10 mM $MgCl_2$) at a m.o.i. of ~0,2 for 15 min at 37°C. After adsorption, the phage/bacteria solutions were transferred to three E-flasks each containing 500 ml LB-medium prewarmed to 37°C. The E-flasks were shaken vigorously at 37°C until lysis occured. Then 2 ml of chloroform was added to the respective flasks and the flasks shaken for additional 10 min. The lysed cultures were subsequently centrifugated at ~15000 g for 40 min and the respective supernatants removed pooled and filtered (0,45 µm nc-filters, Schleicher & Schüll). The filtered media were passed over a column containing IgG-Sepharose 6FF (3 ml sedimented gel which had previously been washed with PBS, pre-eluted with 1 M HAc pH 2,8, washed and equilibrated with PBS). The column was sequencially washed with 30 ml PBS, 60 ml PBS-T and 30 ml distilled H_2O. The bound protein material was eluted with 12 ml 1 M HAc pH 2,8. The eluted fraction was lyophilized and the dried material was dissolved in a TE-buffer (10ml Tris/HCl pH 7,5; 1mM EDTA). SDS-PAGE analysis (a 8-25 % gradient gel run under reducing conditions in the PHAST-system and then stained with Coomassie-blue) showed that the affinity purified material was of high yield and homogeneous in size with a band having the relative migration corresponding to a protein of ~45 kDa (using the low molecular weight marker kit obtained from Bio-Rad). Thus the above described system is well suited to obtain protein MAG. The protein MAG produced in this way had when tested separately all three binding activities ($f\alpha_2M$-, albumin- and IgG-binding activities) .

Example 3 (A). Isolation of a lambda clone originating from *S. equi* supsp *zooepidemicus* strain V which codes for polypeptides possessing α_2M- and IgG-binding activities

A gene library for *S. equi* supsp. *zooepidemicus* strain V was produced in a manner analogous to that described by Frischauf et al., (1983).

Streptococcal DNA was partially digested with Sau3A1 and ligated into BamHI-cleaved lambda EMBL3 vector arms. The ligated DNA was packaged in vitro into phage particles which were then allowed to infect *E. coli* P2392 cells. The resultant phage library was analysed for α_2M- and IgG-binding activity. The resultant phage library was analysed for this purpose on agar plates in soft agar layer which were incubated overnight at 37°C. The next day plates having a plaque frequence of 10^3-10^4 were selected. The plaques from each plate were transferred by replica plating to nc-filters. Transfer was allowed to proceed at room temperature for about 20 min. The filters were subsequently removed and soaked, using gentle agitation, for 30 min in a PBS-T solution (250ml/10 filters with 3 changes of the PBS-T solution in order to remove loosely bound material such as cell debris and components from the growth media). The filters were then sorted into two groups where replicas originating from the same agarplate were represented in each group. The respective group of filters were transferred to a petri dish which either contained approximately 10^7 cpm of ^{125}I-labeled rabbit-IgG-antibodies (specific activity 7 mCi/mg) in PBS-T or approximately 10^7 cpm of ^{125}I-labelled HSA (specific activity 7 mCi/mg) or approximately 10^7 cpm of ^{125}I-labelled fα_2M. After incubation for 2h at room temperature using gentle agitation the respective group of filters were washed separately for 3 x 10 min in 250 ml PBS-T at room temperature (this washing procedure is important to reduce background signals and has to be prolonged if nessecary). After washing the filters were dried and autoradiographed for several days. Analysis of the autoradiogram revealed several plaques reacting with the labelled ligands (α_2M, IgG and HSA). By comparing

autoradiograms corresponding to the different ligands clones binding all three ligands were selected on the original agar plate and replaqued and the binding activties verified in an another two round of binding assay as described above. Finally one clone called "lambda SZG1" expressing all three activities was chosen for the subsequent procedures.

Example 3 (B). Characterization of the lambda SZG1 clone

Purfied phage DNA from the lambda SZG1 clone was analysed by restriction mapping and a preliminary restriction map was constructed. After EcoRI digestion of the lambda SZG1 DNA, fragments were cloned into the pUC19 vector previously cleaved with EcoRI. After ligation and transformation into *E. coli* strain DH5α recombinant clones were screened for expression of $f\alpha_2M$-, and/or IgG-binding activity. This was done as follows clones were grown over night on nc-filters on agar plates. Next day the nc-filters were replica plated to a masterplate whereupon the filters were incubated for 10 min in chloroform vapour in order to release the proteins from the bacterial cells. After washing the filters in large excess of PBS-T (in this step it is important to reduce the bacterial debris attached to the filter to avoid unspecific binding of the labelled ligands used in the next step thereby reducing the background signals) the filters were transferred to petri dishes containing ^{125}I-labelled $f\alpha_2M$ or IgG, respectively as in Example 2 (A) above. After 2h incubation at room temperature under gentle agitation the filters were washed in PBS-T, dried and autoradiographed as mentioned in Example 2 (A). After 2 days of exposure of the filters the autoradiograms were analysed and clones expressing both $f\alpha_2M$-, and IgG-binding activity were isolated. One such clone called pSZG40 harbouring a approximately 2,9 kb EcoRI insert was chosen for further studies.

Example 3 (C). Sequence analysis of the insert of pSZG40 containing the *zag* gene

The nucleotide (nt) sequences of the ~2.9 kb EcoRI inserts of pSZG40 was determined and the nt and deduced amino acid (aa) sequences were compared with earlier published sequences of type III Fc receptor genes (Fahnestock et al., 1986, Guss et al., 1986, Olsson et al., 1987) and the sequences of the *mig* and *mag* genes. This analysis revealed stretches in the *zag* gene homologous to the other mentioned genes. The protein encoded by the *zag* gene is termed protein ZAG.

Example 3 (D) . Localization of the fα_2M-binding domain in protein ZAG.

On the basis of the sequence analysis we used a fusion protein expression system (obtained from Biolabs) to produce various domains of protein ZAG. The 2,9 kb fragment from pSZG40 was purified by preparative agarose gel electrophoresis. The purified fragment was further digested with XmnI and HhaI. After digestion the cleavage mixture was separated on a second preparative agarose gel and a ~500 bp XmnI/HhaI fragment was isolated. The ends of the isolated fragment were converted to blunt ends using T4 DNA polymerase and the fragment was subsequently ligated into pMALC2 (previously cleaved with XmnI). After transformation a clone expressing α_2M-binding was isolated. This clone was called pZAG2. The insert of pZAG2 is shown in Fig 6. Another clone pZAG3 harbouring a ~500bp HhaI/HincII fragment encoding the IgG-binding activity was constructed in the following way. The ~2,9 kb EcoRI fragment mentioned above, was cleaved with HhaI and HincII and the cleavage mixture separated on a preparative agarose gel. The ~500 bp HhaI/HincII fragment was isolated, converted to blunt end with T4 DNA polymerase and cloned into pMALC2 previously cleaved with XmnI. The pZAG3 clone express IgG-binding activity but not fα_2M-binding activity.

Example 3 (E). Purification of protein ZAG using the lambda SZG1 clone

The lambda SZG1 phages were allowed to adsorb to cells of 3 individual tubes containing 3 ml *E coli* P2392 (from an over night culture grown at 37°C in LB-medium with 0.2% maltose and 10 mM $MgCl_2$) at a m.o.i. of ~0,2 for 15 min at 37°C. After adsorption, the phage/bacteria solutions were transferred to three E-flasks each containg 500 ml LB-medium prewarmed to 37°C. The E-flasks were shaken vigorously at 37°C until lysis occured. Then 2 ml of chloroform was added to the respective flasks and the flasks shaken for additional 10 min. The lysed cultures were subsequently centrifugated at ~15000 g for 40 min and the respective supernatants removed pooled and filtered (0,45 um nc-filters, Schleicher & Schüll). The filtered media was passed over a column containing IgG-Sepharose 6FF (3 ml sedimented gel which had previously been washed with PBS, preeluted with 1 M HAc pH 2,8, washed and equilibrated with PBS. The column was sequencially washed with 30 ml PBS, 60 ml PBS-T and 30 ml distilled H_2O. The bound protein material was eluted with 12 ml 1 M HAc pH 2,8. The eluted fraction was lyophilized and the dried material was dissolved in a TE-buffer (10ml Tris/HCl pH 7,5; 1mM EDTA). SDS-PAGE analysis (a 8-25 % gradient-gel run under reducing conditions which was subsequently stained with Coomassie-blue) showed that the affinity purified material was of high yield and homogeneous in size with a band having the relative migration corresponding to a protein of ~45 kDa (using the low molecular weight marker kit obtained from Bio-Rad). Thus the above described system is well suited to obtain protein ZAG.

Conclusions

The binding of the fast form of α_2M to the MIG, MAG and ZAG proteins is mediated by novel sequences extending N-terminally from the IgG-binding domains of the respective proteins. Although large parts of these sequences are dissimilar, cross-inhibition assays have revealed that the α_2M-binding regions of the proteins MIG, MAG and ZAG interact with the same or

closely to each other located domains of the α_2M molecule. The IgG-binding domains alone, produced as fusion proteins, did not show any reactivity with fα_2M. The highly homologous recombinant protein G used in control experiments similarly did not react with the α_2M but only with the IgG. Thus, the only binding activity of the ~70 amino acid IgG-binding segments in protein MIG, MAG and ZAG and the corresponding segments in protein G from strain G148 is the binding of IgG. These results argue against earlier data published by Sjöbring et al.(1989), that the IgG domains of protein G also bind α_2M.

After the submissiom of the first patent application on the 6th of september 1993 (Nr. 9302855-3, the priority claim), more extended data of the three α_2M-binding proteins described above (see examples 1-3) have been published. For a detailed description of the protein MIG and its gene see reference Jonsson and Müller, 1994, for protein MAG the key reference is Jonsson et al., 1994. The protein ZAG and its gene as well as a comparison of all 3 α_2M-binding proteins have been described in the thesis of Hans Jonsson: "Cell surface proteins of animal group G streptococci mediating binding to plasma proteins", Uppsala 1994.

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Legends to the figures

Fig. 1. Restriction map of the Hind III restriction fragment containing the *mig* gene from *Streptococcus dysgalactiae*, strain SC1 and schematic drawings of the various fusion protein products. The upper line indicates the position of the restriction sites used in the construction of the various expression clones. The upper bar represents the native MIG protein with the binding activities indicated. The other three bars represent the protein products encoded by the expression clones pMIG 1-3.

Fig. 2. Western Blot of fusion proteins. The fusion proteins were constructed as indicated in Fig. 2 and produced in *E.coli* strain DH5a. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and subsequently analysed for binding activity with α_2M (bovine α_2M, fast form, peroxidase conjugate) and IgG (goat IgG peroxidase conjugate). Lane 1, recombinant protein G as a control; Lane 2, lysate of clone pMIG3 encoding the 5 IgG-binding regions of protein MIG; Lane 3, lysate of clone pMIG2 encoding the α_2M-binding site; and Lane 4, lysate of clone pMIG1 encoding essentially the native protein MIG.

Fig. 3. Inhibition of binding of α_2M (A) and IgG (B) to *Streptococcus dysgalactiae*, strain SC1 by the fusion proteins encoded by pMIG2 and pMIG3. The purified gene products of pMIG2 and pMIG3 encoding the α_2M-binding and IgG-binding domains of protein MIG, respectively, were tested in a microtiter plate inhibition assay. SC1 cells were immobilised on the plates and the binding of labelled ligands was recorded in the presence of increasing amounts of fusion protein. The hatched bar indicates the binding in the absence of fusion protein.

Figure 4. Nucleotide sequence of the *mag* gene from *S. dysgalactiae* strain 8215 and the deduced aa sequence. Underlined, the putative transcription initiation signals. The ribosome binding site is double underlined. The start of the signal sequence (S), α_2M-, Albumin- (Alb) and IgG-binding domains, respectively, are indicated as well as the cell wall binding (W) and membrane spanning (M) regions. In the C-terminal the LPXTGX motif is underlined. The nucleotide sequence was determined for both DNA strands by the dideoxy chain-termination method of Sanger et al.(1977). The juxtaposition in lambda SD1 of the inserts in pSD100 and pSD101 was verified using oligonucleotide primers (hybridizing on both sides of the HindIII site) allowing sequencing over the *Hin*dIII site in position 862. Sequence reactions were performed using "Sequenase, version 2.0" kit (United States Biochemical Corporation, Cleveland, Ohio, USA). The obtained nucleotide sequences were analyzed using the PC/GENE computer software package, Intelligenetics Inc. CA, USA.

Figure 5. (A) Schematic presentation of protein products coded by the expression clones pMAG 1-4 derived from *S. dysgalactiae*, strain 8215. Restriction sites used in the construction work are indicated. The two upper lines represent inserts of streptococcal DNA in pSD100 and pSD101. The upper bar represents protein MAG.(The Mal E portion is not drawn to scale).

(B) SDS-PAGE of total cell lysates from clones harbouring pMAG1-4. Lanes: (M) molecular size markers, lanes 1-4 correspond to lysates from *E. coli* pMAG1-4,respectively. **Methods:** After IPTG induction the cells were harvested, lysed and protein samples were subjected to SDS-PAGE by the method of Laemmli (1970) using a 4% spacer and 12% separation gel. After electrophoresis the gel was stained with Coomassie Brilliant Blue, destained and photographed.

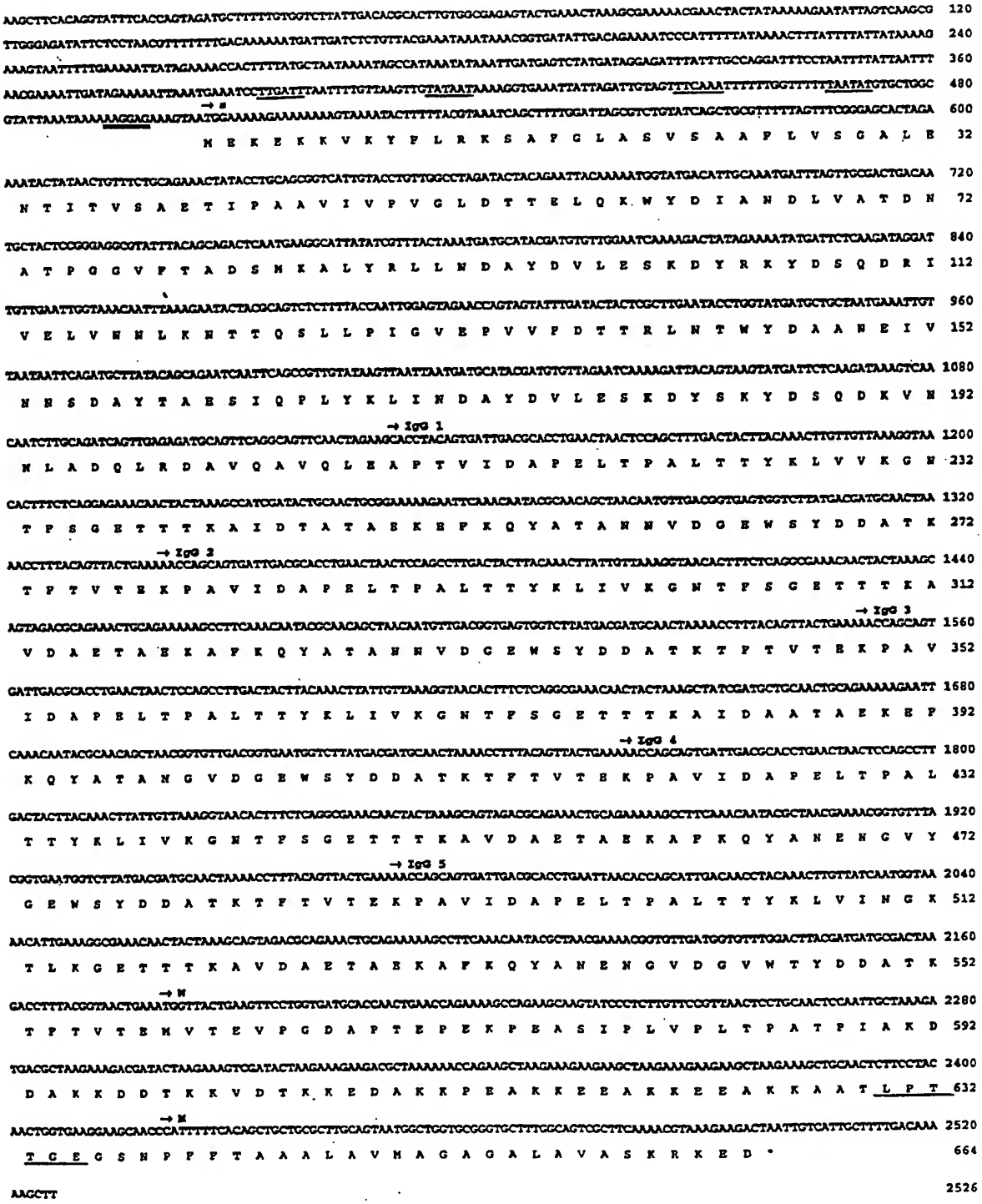
(C) Western-blot analysis of the proteins encoded by pMAG1-4. Three parallel gels were run using the same conditions as described in the legend to Figure 5(B). After the electrophoresis the proteins were electrophoretically transfered to nitrocellulose membranes. The membranes were

washed and blocked in a PBS-T solution and separately incubated with horseradish peroxidase labelled bovine fα_2M, goat albumin and polyclonal goat IgG, respectively. After incubation for one hour at room temperature the membranes were washed with PBS-T and the bound labelled serum proteins were visualized by the addition of a 4-chloro-1-naphtol solution.

Figure 6. Nucleotide sequence and deduced amino acid sequence of the *zag* gene (from Streptococcus zooepidemicus)encoding fα_2M-binding activity.

Annexed information to the following page:

Page 31 shows the nucleotide sequence and deduced amino acid sequence of the cloned *mig* gene from *Streptococcus dysgalactiae,* strain SC1. Features of the sequence are indicated as follows: underlined nucleotide sequences represent possible promoter signals, double underlined nucleotide sequence represents the ribosome binding site, underlined amino acid sequence marks a hexapeptide matching the putative wall anchoring LPXTGX consensus, s represents the start of the signal sequence, IgG1-IgG5 represent the IgG-binding, 70 aa long repeat units, W represents the cell wall spanning region, M represents the membrane spanning domain.



SUBSTITUTE SHEET

Claims

1. Recombinant DNA molecule containing a nucleotide sequence which codes for a protein or polypeptide having fα_2M-binding activity as protein MAG.

2. Plasmid or phage containing a nucleotide sequence which codes for a protein or polypeptide having the same fα_2M-binding activity as protein MAG.

3. Microorganism containing at least one recombinant DNA molecule according to claim 1.

4. Microorganism containing at least one plasmid or phage according to claim 2.

5. Method for producing protein MAG or a polypeptide thereof, characterized in that
- at least one recombinant DNA molecule according to claim 1 is inserted in a microorganism,
- said microorganism is cultured in a suitable medium,
- the protein thus formed is isolated by affinity chromatographic purification using an immobilised ligand.

6. Recombinant DNA molecule according to claim 1, characterized by containing one or more of the following nucleotide sequence:

```
        10         20         30         40         50         60
CTGCGTTTTTAGTTGGGACTGCGGTAGTAAATGCGGAAGAGTCAACTGTTTCGCCTGTGA
  A  F  L  V  G  T  A  V  V  N  A  E  E  S  T  V  S  P  V

        70         80         90        100        110        120
CAGTTGCTACAGATGCAGTTACTACTTCTAAGGAAGCGCTTGCGATAATTAACAAGCTAA
T  V  A  T  D  A  V  T  T  S  K  E  A  L  A  I  I  N  K  L

       130        140        150        160        170        180
GTGAAGATAATTTAAATAATCTTGACATCCAGGAAGTATTGGCCAAAGCGGGGAGGGACA
S  E  D  N  L  N  N  L  D  I  Q  E  V  L  A  K  A  G  R  D

       190        200        210        220        230        240
TTTTAGCCTCTGACTCAGCAGATACTATCAAAGCACTTCTTGCTGAAGTTACCGCTGAAG
I  L  A  S  D  S  A  D  T  I  K  A  L  L  A  E  V  T  A  E

       250        260        270        280        290        300
TTACTCGTTTGAATGAGGAAAAGATGGCACGTGATGCAGTAGACAAAGCTATTGCAGCAG
V  T  R  L  N  E  E  K  M  A  R  D  A  V  D  K  A  I  A  A

       310        320        330        340        350        360
ATGCAGCCGCTTTTTCTGAATTAAAAGATGCTCAACTGAAAGCATATGAAGATCTTGCGA
D  A  A  A  F  S  E  L  K  D  A  Q  L  K  A  Y  E  D  L  A

       370        380        390        400        410        420
AACTCGCAGCAGATACAGACTTAGATTTAGATGTTGCTAAAATTATAAATGACTACACTA
K  L  A  A  D  T  D  L  D  L  D  V  A  K  I  I  N  D  Y  T

       430        440        450        460        470        480
CAAAAGTTGAAAATGCAAAAACAGCAGAAGATGTTAAAAAAAATTTTTGAAGAATCTCAAA
T  K  V  E  N  A  K  T  A  E  D  V  K  K  I  F  E  E  S  Q

       490        500
ATGAAGTGACACGTATTAAAACAGAAAA
N  E  V  T  R  I  K  T  E
```

7. Plasmid or phage containing one or more nucleotide sequences according to claim 6.

8. Microorganism containing at least one plasmid or phage according to claim 7.

9. The use of protein MAG or fragments thereof to inhibit the binding of fα_2M to streptococcal cells.

10. Recombinant DNA molecule containing a nucleotide sequence which codes for a protein or polypeptide having fα_2M binding activity as protein MIG.

11. Plasmid or phage containing a nucleotide sequence which codes for a protein or polypeptide having the same fα_2M binding activity as protein MIG.

12. Microorganism containing at least one recombinant DNA molecule according to claim 10.

13. Microorganism containing at least one plasmid or phage according to claim 11.

14. Method for producing protein MIG or a polypeptide thereof, characterized in that
- at least one recombinant DNA molecule according to claim 10 is inserted in a microorganism,
- said microorganism is cultured in a suitable medium,
- the protein thus formed is isolated by affinity chromatographic purification using an immobilised ligand.

15. Recombinant DNA molecule according to claim 10, characterized by containing one or more of the following nucleotide sequence:

```
        10         20         30         40         50         60
CTGCGTTTTTAGTTTCGGGAGCACTAGAAAATACTATAACTGTTTCTGCAGAAACTATAC
  A  F  L  V  S  G  A  L  E  N  T  I  T  V  S  A  E  T  I

        70         80         90        100        110        120
CTGCAGCGGTCATTGTACCTGTTGGCCTAGATACTACAGAATTACAAAAATGGTATGACA
P  A  A  V  I  V  P  V  G  L  D  T  T  E  L  Q  K  W  Y  D

       130        140        150        160        170        180
TTGCAAATGATTTAGTTGCGACTGACAATGCTACTCCGGGAGGCGTATTTACAGCAGACT
I  A  N  D  L  V  A  T  D  N  A  T  P  G  G  V  F  T  A  D

       190        200        210        220        230        240
CAATGAAGGCATTATATCGTTTACTAAATGATGCATACGATGTGTTGGAATCAAAAGACT
S  M  K  A  L  Y  R  L  L  N  D  A  Y  D  V  L  E  S  K  D

       250        260        270        280        290        300
ATAGAAAATATGATTCTCAAGATAGGATTGTTGAATTGGTAAACAATTTAAAGAATACTA
Y  R  K  Y  D  S  Q  D  R  I  V  E  L  V  N  N  L  K  N  T

       310        320        330        340        350        360
CGCAGTCTCTTTTACCAATTGGAGTAGAACCAGTAGTATTTGATACTACTCGCTTGAATA
T  Q  S  L  L  P  I  G  V  E  P  V  V  F  D  T  T  R  L  N

       370        380        390        400        410        420
CCTGGTATGATGCTGCTAATGAAATTGTTAATAATTCAGATGCTTATACAGCAGAATCAA
T  W  Y  D  A  A  N  E  I  V  N  N  S  D  A  Y  T  A  E  S

       430        440        450        460        470        480
TTCAGCCGTTGTATAAGTTAATTAATGATGCATACGATGTGTTAGAATCAAAAGATTACA
I  Q  P  L  Y  K  L  I  N  D  A  Y  D  V  L  E  S  K  D  Y

       490        500        510
GTAAGTATGATTCTCAAGATAAAGTCAACAATCTTGCAG
S  K  Y  D  S  Q  D  K  V  N  N  L  A
```

16. Plasmid or phage containing one or more nucleotide sequences according to claim 15.

17. Microorganism containing at least one plasmid or phage according to claim 16.

18. The use of protein MIG or fragments thereof to inhibit the binding of $f\alpha_2M$ to streptococcal cells.

19. Recombinant DNA molecule containing a nucleotide sequence which codes for a protein or polypeptide having $f\alpha_2M$ binding activity as protein ZAG.

20. Plasmid or phage containing a nucleotide sequence which codes for a protein or polypeptide having the same $f\alpha_2M$ binding activity as protein ZAG.

21. Microorganism containing at least one recombinant DNA molecule according to claim 19.

22. Microorganism containing at least one plasmid or phage according to claim 20.

23. Method for producing protein ZAG or a polypeptide thereof, characterized in that
- at least one recombinant DNA molecule according to claim 19 is inserted in a microorganism,
- said microorganism is cultured in a suitable medium,
- the protein thus formed is isolated by affinity chromatographic purification using an immobilised ligand.

24. Recombinant DNA molecule according to claim 19, characterized by containing one or more of the following nucleotide sequence:

```
        10         20         30         40         50         60
TCTTCAGTGGGTGCTCTAGATGCTACAACGGTGTTAGAGCCTACAACAGCCTTCATTAGA
 S  S  V  G  A  L  D  A  T  T  V  L  E  P  T  T  A  F  I  R

        70         80         90        100        110        120
GAGGCTGTTAGGGAAATCAATCAGCTTAGTGATGACTACGCTGACAATCAAGAGCTTCAG
 E  A  V  R  E  I  N  Q  L  S  D  D  Y  A  D  N  Q  E  L  Q

       130        140        150        160        170        180
GCTGTTCTTGCTAATGCTGGAGTTGAGGCACTTGCTGCAGATACTGTTGATCAAGCCAAA
 A  V  L  A  N  A  G  V  E  A  L  A  A  D  T  V  D  Q  A  K

       190        200        210        220        230        240
GCAGCTCTTGACAAAGCAAAGGCAGCTGTTGCTGGTGTTCAGCTTGATGAAGCAAGACGT
 A  A  L  D  K  A  K  A  A  V  A  G  V  Q  L  D  E  A  R  R

       250        260        270        280        290        300
GAGGCTTACAGAACAATCAATGCCTTAAGTGATCAGCACGAAAGCGATCAAAAGGTTCAG
 E  A  Y  R  T  I  N  A  L  S  D  Q  H  E  S  D  Q  K  V  Q

       310        320        330        340        350        360
CTAGCTCTAGTTGCTGCAGCAGCTAAGGTGGCAGATGCTGCTTCAGTTGATCAAGTGAAT
 L  A  L  V  A  A  A  A  K  V  A  D  A  A  S  V  D  Q  V  N

       370        380        390        400        410        420
GCAGCCATTAATGATGCTCATACAGCTATTGCGGACATTACAGGAGCAGCCTTGTTGGAG
 A  A  I  N  D  A  H  T  A  I  A  D  I  T  G  A  A  L  L  E

       430        440        450        460        470        480
GCTAAAGAAGCTGCTATCAATGAACTAAAGCAGTATGGCATTAGTGATTACTATGTGACC
 A  K  E  A  A  I  N  E  L  K  Q  Y  G  I  S  D  Y  Y  V  T

       490        500        510
TTAATCAACAAAGCCAAAACTGTTGAAGGTGTCAATGCG
 L  I  N  K  A  K  T  V  E  G  V  N  A
```

25. Plasmid or phage containing one or more nucleotide sequences according to claim 24.

26. Microorganism containing at least one plasmid or phage according to claim 25.

27. The use of protein ZAG or fragments thereof to inhibit the binding of fα_2M to streptococcal cells.

28. The use of proteins MIG, MAG or ZAG to inhibit the interaction of said proteins with the fast form of α_2-macroglobulins.

Figure 1.

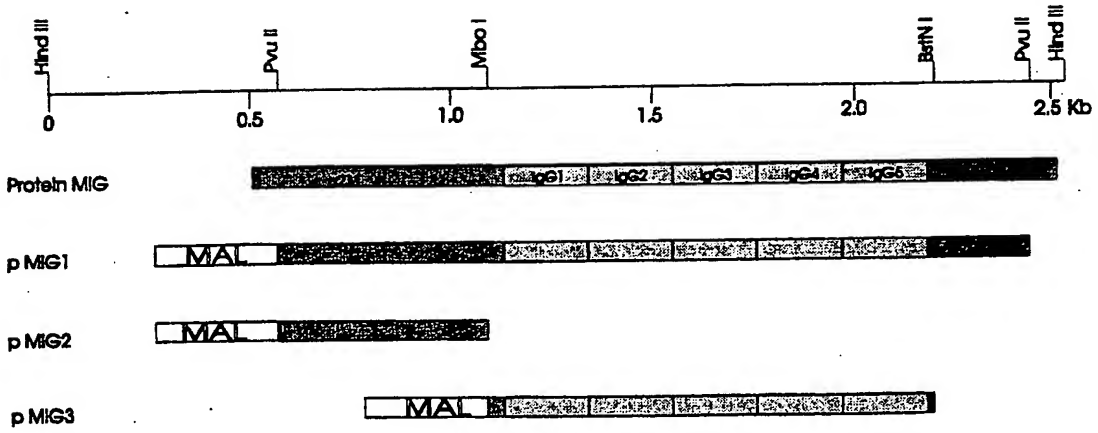

Hind III
Pvu II
Mbo I
BstN I
Pvu II
Hind III
0
0.5
1.0
1.5
2.0
2.5 Kb
Protein MIG
gG1
gG2
gG3
gG4
gG5
p MIG1
MAL
p MIG2
MAL
p MIG3
MAL

Figure 2.

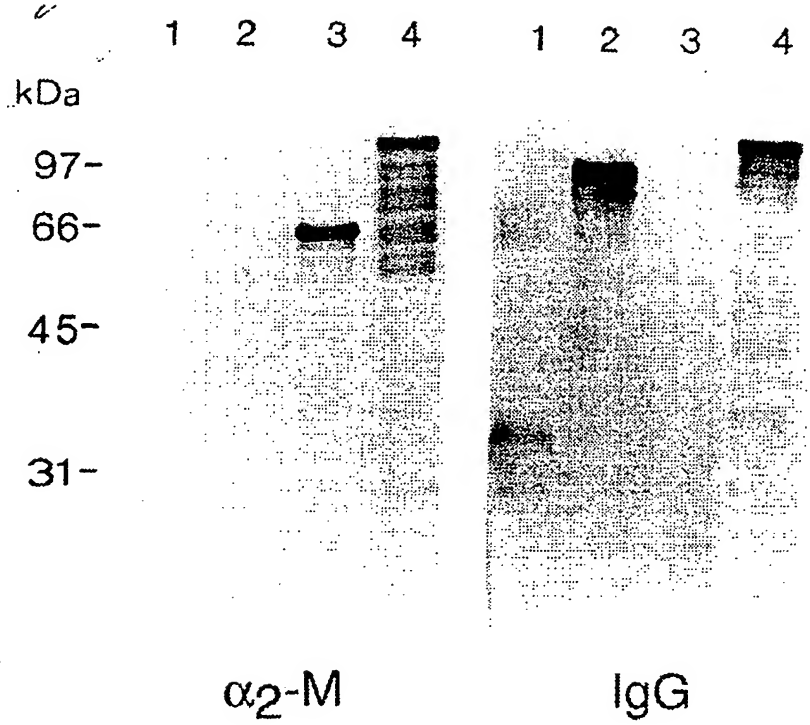
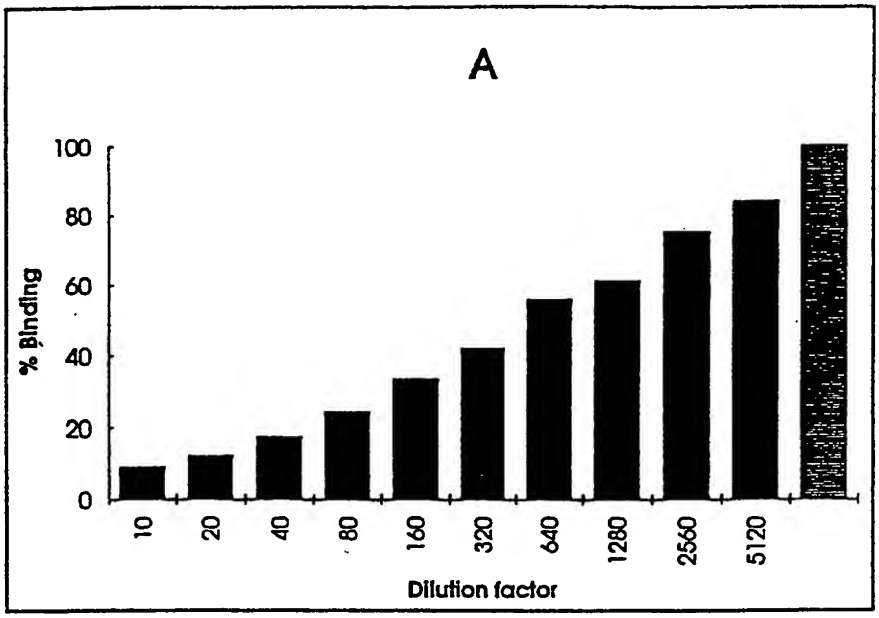


Figure 3.


A
100
80
60
40
20
0
% Binding
10
20
40
80
160
320
640
1280
2560
5120
Dilution factor

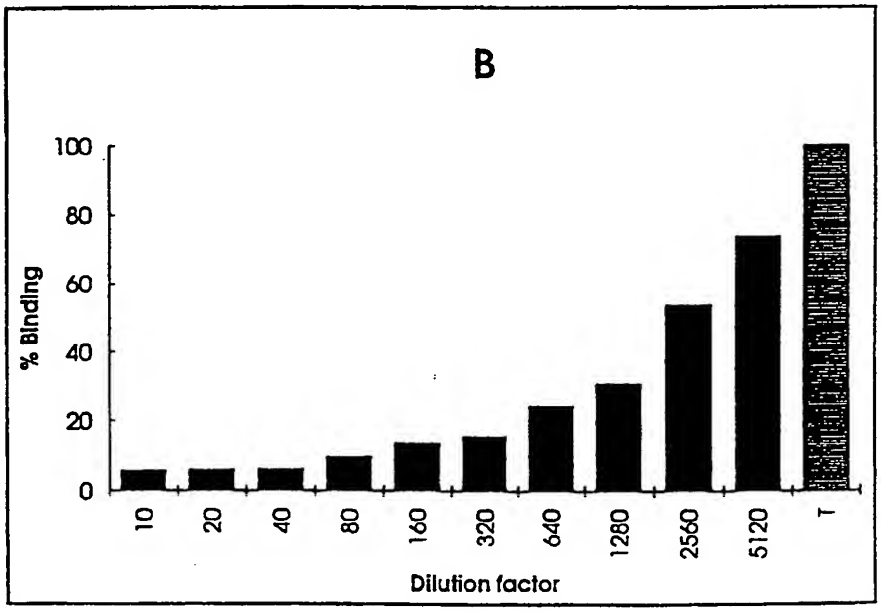
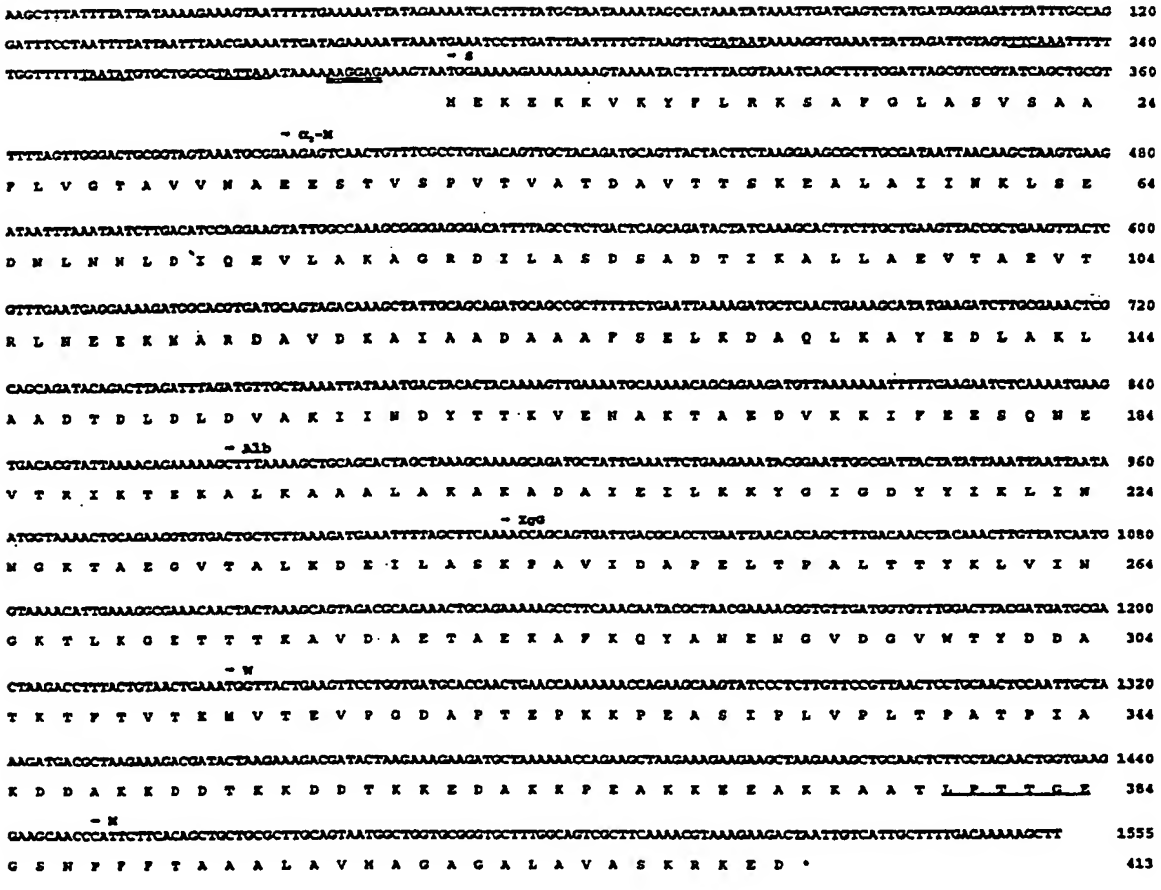

B
100
80
60
40
20
0
% Binding
10
20
40
80
160
320
640
1280
2560
5120
T
Dilution factor

Figure 4.



SUBSTITUTE SHEET

Figure 5A.

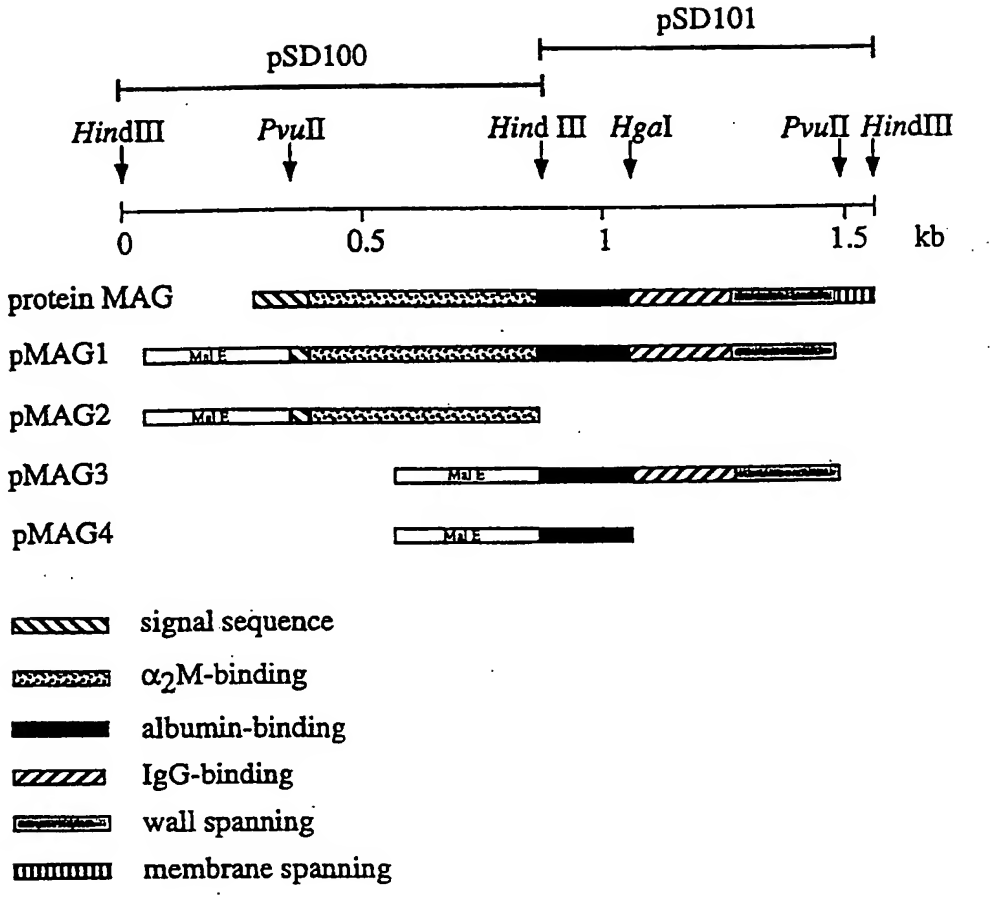

pSD101
pSD100
HindIII
PvuII
Hind III
HgaI
PvuII
HindIII
0
0.5
1
1.5
kb
protein MAG
pMAG1
pMAG2
pMAG3
pMAG4
signal sequence
α2M-binding
albumin-binding
IgG-binding
wall spanning
membrane spanning

Figure 5B.

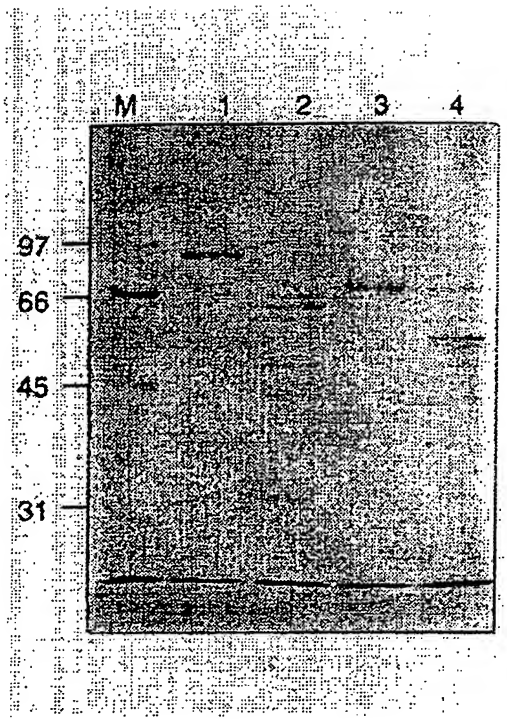
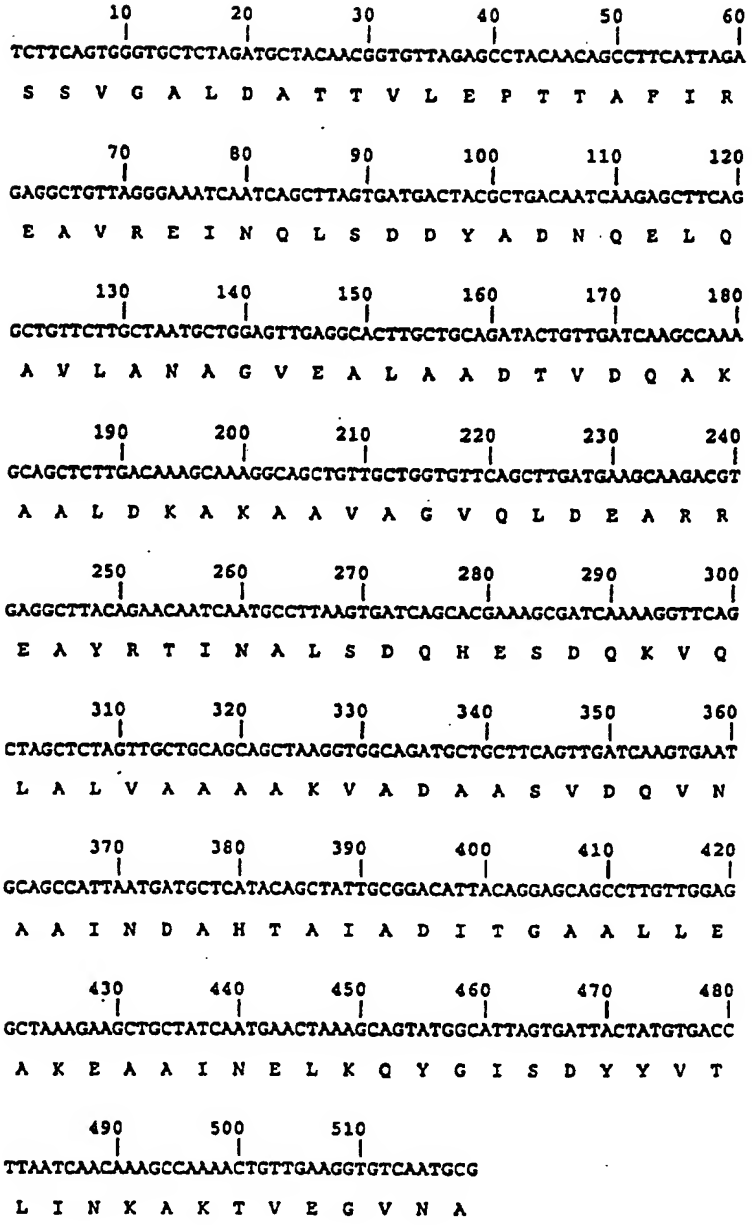


Figure 5C.

Figure 6.


10 20 30 40 50 60
TCTTCAGTGGGTGCTCTAGATGCTACAACGGTGTTAGAGCCTACAACAGCCTTCATTAGA
S S V G A L D A T T V L E P T T A F I R
70 80 90 100 110 120
GAGGCTGTTAGGGAAATCAATCAGCTTAGTGATGACTACGCTGACAATCAAGAGCTTCAG
E A V R E I N Q L S D D Y A D N Q E L Q
130 140 150 160 170 180
GCTGTTCTTGCTAATGCTGGAGTTGAGGCACTTGCTGCAGATACTGTTGATCAAGCCAAA
A V L A N A G V E A L A A D T V D Q A K
190 200 210 220 230 240
GCAGCTCTTGACAAAGCAAAGGCAGCTGTTGCTGGTGTTCAGCTTGATGAAGCAAGACGT
A A L D K A K A A V A G V Q L D E A R R
250 260 270 280 290 300
GAGGCTTACAGAACAATCAATGCCTTAAGTGATCAGCACGAAAGCGATCAAAAGGTTCAG
E A Y R T I N A L S D Q H E S D Q K V Q
310 320 330 340 350 360
CTAGCTCTAGTTGCTGCAGCAGCTAAGGTGGCAGATGCTGCTTCAGTTGATCAAGTGAAT
L A L V A A A A K V A D A A S V D Q V N
370 380 390 400 410 420
GCAGCCATTAATGATGCTCATACAGCTATTGCGGACATTACAGGAGCAGCCTTGTTGGAG
A A I N D A H T A I A D I T G A A L L E
430 440 450 460 470 480
GCTAAAGAAGCTGCTATCAATGAACTAAAGCAGTATGGCATTAGTGATTACTATGTGACC
A K E A A I N E L K Q Y G I S D Y Y V T
490 500 510
TTAATCAACAAAGCCAAAACTGTTGAAGGTGTCAATGCG
L I N K A K T V E G V N A

INTERNATIONAL SEARCH REPORT

[redacted]

International application No.
PCT/S[redacted]/00826

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/315, C07K 14/735, C12N 15/11
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, WPI, CLAIMS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, file 155, MEDLINE, Dialog accession no. 06305921, Medline accession no. 87279921, Chhatwal GS et al: "Novel complex formed between a nonproteolytic cell wall protein of group A streptococci and alpha 2-macroglobulin", & J Bacteriol Aug 1987, 169 (8) p3691-5	6-8
A	Dialog Information Services, file 5, BIOSIS PREVIEWS, Dialog accession no. 7363817, Biosis no. 89014836, SJOBRING U et al: "IG-binding bacterial proteins also bind proteinase inhibitors", & J IMMUNOL 143 (9). 1989. 2948-2954	6-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 February 1995	13 -02- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Jonny Brun Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/S[redacted]/00826

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-5, 9, 28
because they relate to subject matter not required to be searched by this Authority, namely:

Since "the protein MAG" is not clearly defined, the wording "having $f\alpha_2$M-binding activity as protein MAG" according to claims 1-5, 9 and the use of protein "MAG" according to claims 1-5,9 and the use of protein "MAG" according to claim 28 are not considered clear and concise (PCT Art. 6).

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-9, 28

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

1. Claims 1-9 (completely) and claim 28 (partially) relate to a protein or polypeptide having fα_2M-binding activity as protein MAG.
2. Claims 10-18 (completely) and claim 28 (partially) relate to a protein or polypeptide having fα_2M-binding activity as protein MIG.
3. Claims 19-27 (completely) and claim 28 (partially) relate to a protein or polypeptide having fα_2M-binding activity as protein ZAG.

As it stated in Annex B to Administrative Instructions under the PCT, in force July 1, 1992 (PCT) GAZETTE 1992, June 25, pages 7062-9, (see page 7063 and example 5) unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same corresponding "special technical features" -i.e. features that define a contribution which each of the inventions makes over the prior art.

The unifying feature of group 1, 2 and 3 are streptococcal proteins having fα_2M-binding activity.

From i) Dialog Information Services, File 155: Medline, Dialog accession no. 06305921 & Chhatwal GS et al, J Bacteriol (US), 169 (8) p 3691-5 and ii) Dialog Information Services, File 5: Biosis, Dialog accession no. 7363817 & Sjöbring U et al, J Immunol 143 (9), 1989, 2948-2954 are streptococcal proteins having α_2M-binding activity known.

Thus, the unifying feature of group 1, 2 and 3 are not a "special technical feature" and, a posteriori, the application is considered to comprise three separate inventions.

Consequently, the search has been restricted to the invention first mentioned, which relates to a protein or polypeptide having fα_2M-binding activity as protein MAG.